THE RÔLE OF METAL IONS IN THE ENZYMATIC HYDROLYSIS OF ARGININE. ACTIVATION OF ENZYMES

IV. THE JACK BEAN ARGININOLYTIC ENZYME
V. THE SPECIFICITY OF GUANIDINO COMPOUNDS. ACTIVATING METAL IONS AND LIVER ARGINASE

A DISSERTATION

SUBMITTED TO THE BOARD OF UNIVERSITY STUDIES OF THE JOHNS HOPKINS UNIVERSITY IN CONFORMITY WITH THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

C. CHESTER STOCK

BALTIMORE

1937

REPRINTED FROM
THE JOURNAL OF BIOLOGICAL CHEMISTRY
VOL. 125, NO. 2, PAGES 753–793, 1938

THE RÔLE OF METAL IONS IN THE ENZYMATIC HYDROLYSIS OF ARGININE. ACTIVATION OF ENZYMES

IV. THE JACK BEAN ARGININOLYTIC ENZYME
V. THE SPECIFICITY OF GUANIDINO COMPOUNDS. ACTIVATING METAL IONS AND LIVER ARGINASE

A DISSERTATION

SUBMITTED TO THE BOARD OF UNIVERSITY STUDIES OF THE JOHNS HOPKINS UNIVERSITY IN CONFORMITY WITH THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

C. CHESTER STOCK

BALTIMORE
1937

REPRINTED FROM
THE JOURNAL OF BIOLOGICAL CHEMISTRY
VOL. 125, NO. 2, PAGES 753-793, 1938

ACKNOWLEDGMENT

I wish to express here my appreciation to each member of the staff of the Department of Physiological Chemistry of the Johns Hopkins School of Medicine for all his kindnesses and instruction. I desire especially to thank Dr. W. Mansfield Clark for the privilege and advantage of studying in the department under his guidance; Dr. Leslie Hellerman for his considerate and stimulating direction of my research; and Miss Marie E. Perkins for assistance in numerous analyses. I also wish to thank Dr. Robert D. Stiehler of the Johns Hopkins Hospital for the use of his glass electrode.

R. UNIV.
BIBLIOTHEEK
LEIDEN

ACTIVATION OF ENZYMES

IV. THE JACK BEAN ARGININOLYTIC ENZYME

By C. CHESTER STOCK

(From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore)

(Received for publication, July 16, 1938)

In connection with a study of the rôle of certain metal ions as "activators" of liver arginase, Hellerman and Perkins presented evidence (1) for the existence of an enzyme in the jack bean which accelerates the hydrolysis of *d*-arginine. This catalyst at present may be referred to as jack bean arginase. Its activity, like that of liver arginase, was found to be enhanced significantly under well defined conditions by cobaltous ion or by certain other heavy metal ions. The decomposition of *d*-arginine in the presence of the activated enzyme and of accompanying urease yielded, after a sufficient reaction time, almost 2 equivalents of ammonia.

The description of the initial experiments ((1) pp. 189, 190) brought out that relatively *crude* preparations of jack bean urease, including crude crystalline urease, had been observed to possess argininolytic properties. Highly recrystallized urease (*e.g.*, thrice recrystallized or more) was not used. There is no present reason to assign any catalytic activity other than the ureolytic to the highly recrystallized product, the identity of which as a homogeneous protein is suggested by several lines of evidence. Therefore, the reference to crystalline protein urease in the paper by Hellerman and Perkins was not to have been construed as implying that the urease molecule itself, as represented by Sumner's highly recrystallized preparation, may in the presence of cobaltous ion, etc., assume argininolytic properties. Actually, recrystalliza-

tions of urease appear to eliminate progressively the arginase activity[1] (*cf.* (2)). The crude crystallized preparations provided a convenient source of jack bean arginase, activatable by appropriate heavy metal ions. The similarities in certain characteristics of the jack bean and liver argininolytic enzymes offer interesting suggestions concerning the nature of the arginine-arginase reaction.

The evidence underlying the working hypothesis that *liver* arginase may consist of a metallo complex has been cited elsewhere (1, 3). It may be considered that a more or less separable metallic component is bound, presumably by coordinate bonds, to "donor" groups of a non-metallic part, probably of protein nature. It has been indicated further that the substitution of a constituent of the jack bean for the donor component of liver arginase and of such an ion as cobaltous for the metallic portion, in the enzymatic hydrolysis of *d*-arginine, suggests in a sense the construction of an artificial arginase (3). If, moreover, metalloenzyme be assumed to have combined with *d*-arginine in the formation of a dissociable enzyme-substrate complex, a primary factor in the control of stability of the resulting complex molecule might reside in the coordinative linking of the enzyme's metal ion to the substrate's α-amino group. Such a process virtually would involve a competition between enzyme-metal ion and hydrogen ion for the substrate's α-amino group in the pH range in which arginase is active. This is consistent with the following. From the magnitude of arginine's several dissociation constants (4) it follows that the zwitter ion of isoelectric arginine, $^{+}[H_2N{=}C(NH_2){-}N(H)]{-}(CH_2)_3{-}C(H)(NH_2){-}COO^{-}$, involves essentially the guanidinium cation and the carboxyl anion. In a titration of arginine from its isoelectric point to a region of lowered pH (*e.g.*, pH 6.5), there is concerned chiefly the conversion of the α-amino group to the α-ammonium configuration ($-NH_2 \rightarrow -NH_3^{+}$). The work of Hunter (5) and of Edlbacher (6, 7) and their coworkers with pH-activity curves shows that liver arginase functions in a wide pH range (roughly pH 5 to 11). The liver preparation used by Hellerman and Perkins appears to contain material characteristic of native arginase, together with some inactive enzyme which assumes argininolytic activity in the

[1] Perkins, M. E., unpublished experiments.

presence of certain salts. If our concepts relative to arginase have any validity, it must be anticipated that the addition of activating metal ion to arginine-arginase mixtures such as we have used should alter significantly the pH-activity curve. The argument may be extended *mutatis mutandis* to the behavior of jack bean arginase.

This paper records exploratory observations upon the relation between activity and pH for jack bean arginase and upon modifications introduced by the presence of cobaltous, nickelous, or manganous salts, while the following paper gives similar data for the liver enzyme. There is presented also a brief account of the kinetics of *d*-arginine hydrolysis in the presence of jack bean arginase and cobaltous ion. In addition, there are included some experiments which illustrate the distortion of the titration curve of arginine in the presence of cobaltous, nickelous, or manganous ions and show in a preliminary way the relative abilities of these ions *alone* to combine with the substrate amino acid.

EXPERIMENTAL

Activity-pH Curves of Jack Bean Argininolytic Enzyme — The characteristics have been determined on the basis of the extent of hydrolysis of *d*-arginine in a given time, rather than by the ideal method of comparing the initial reaction velocities. The procedure was considered admissible, since for comparative purposes only the main characteristics of the several curves were of interest. Pertinent is the demonstration by Hunter and Morrell, working with certain preparations of liver arginase, that curves so obtained are roughly similar to those given by a more rigorous method.

Determinations were carried out as follows: To a solution (contained in a 25 ml. volumetric flask of resistant glass) composed of 10 ml. of buffer (see below), 46 mg. of *d*-arginine hydrochloride, and appropriate heavy metal salt were added caprylic alcohol (2 drops), 5 ml. of a solution containing 0.1 gm. of crude jack bean preparation (8) or crude crystalline (9) urease of comparable argininolytic activity, and redistilled water to make 20 ml. The mixture was kept for $2\frac{1}{2}$ hours at 30°, after which there was added 1 ml. of 0.25 M sulfuric acid and the solution was heated 10 minutes at 100°; it was cooled and diluted to 25 ml. After filtration, ammonia in 10 ml. was determined by the usual aeration method

(*cf.* (1)) and estimated in terms of ml. of 0.0200 N HCl neutralized. Appropriate controls were carried out upon reagents and the heat-denatured enzyme. Water redistilled in glass was used in all enzyme work.

When activating metal salt was employed, there were present 3 mg. of $CoCl_2 \cdot 6H_2O$, 5 mg. of $NiCl_2 \cdot 6H_2O$, or 3 mg. of $MnCl_2 \cdot 4H_2O$.

The buffers used were acetate, phosphate, and glycine, respectively, for the ranges of pH 4.5 to 5.5, 5.7 to 7.8, and 8.2 to 12.6. The phosphate buffers (10) were used so that the *ionic strength* in the reaction mixture was 1. The glycine buffers were used with approximately the same ionic strength, while acetate buffers were employed in a final concentration of 1.0 M with respect to total acetate. The pH of each buffer at the proper dilution was determined by means of a hydrogen electrode. Since the buffer salts may in varying degree display characteristic specific effects, some care must be taken in the comparison of results obtained with different kinds of buffers. The more significant changes resulting from the presence of the activating metal ions occur in the range covered by the phosphate buffers; primary consideration is to be given to the data obtained in this range.

Fig. 1 gives the mean activity-pH curve for jack bean arginase and records, in addition, the data obtained when there was present cobaltous, nickelous, and manganous salt, respectively. Each curve is based upon a number of independent determinations of the relative activities through a range of pH from 4.5 to 12.6 at intervals of 0.05 to 0.50 pH unit, the magnitude of the intervals depending upon the degree of change of slope of the curve (*cf.* (11)).

Kinetics of d-Arginine Hydrolysis in Presence of Jack Bean Arginase and Cobaltous Ions — A suitable rate study promised to throw light upon the action of the argininolytic enzyme and upon the part played by *urease* in the over-all process. The results will be discussed in another section.

After considerable preliminary work, there were found the conditions under which consistent, reproducible results were obtainable. Concentrations of certain reagents greater than those used in earlier work were found essential. Especially important was the use of more concentrated buffers, since under

the conditions (pH 7.5) a small change in pH affects appreciably the activity of cobalt-enzyme (*cf.* Fig. 1). The data reported here are from one of five similar runs, all of which yielded results which fit the simple first order relation. The values of the several average reaction constants obtained differed somewhat owing to variations in the enzyme content of the preparations used.

Under the conditions of the present observations the urease contained in the jack bean preparations used has been found to effect in less than 1 minute the hydrolysis of a quantity of urea

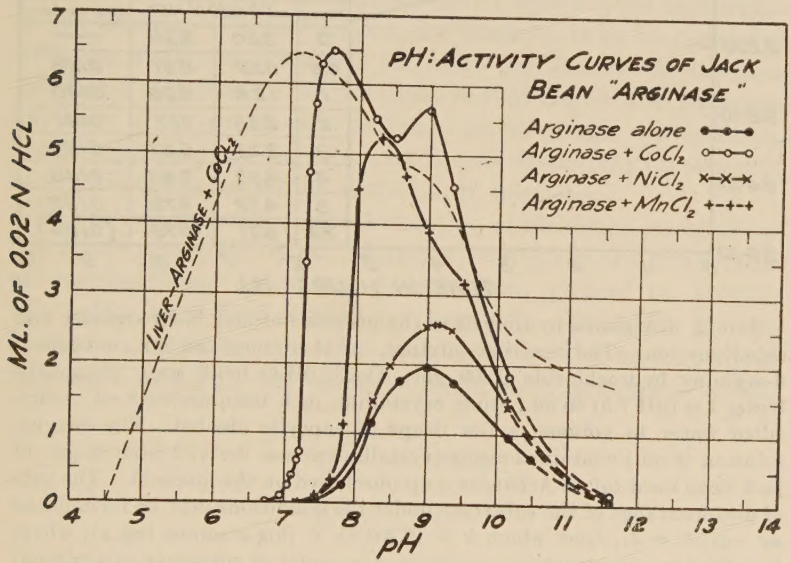
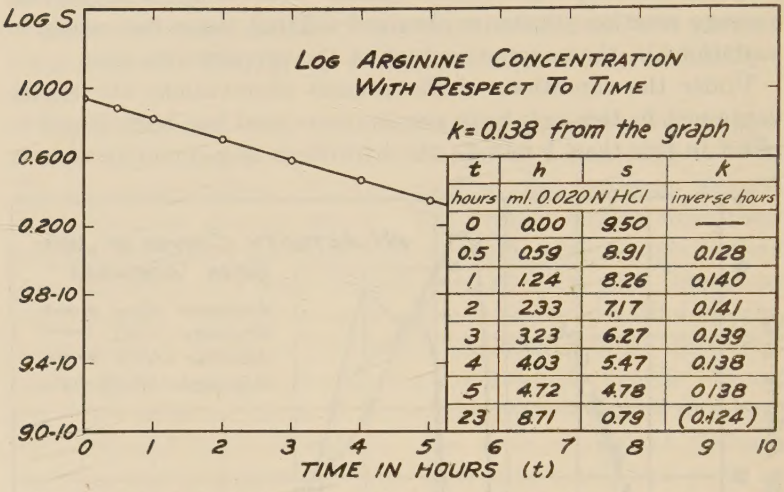


Fig. 1

equivalent to the total amount of the *d*-arginine originally present in the reaction mixtures. This is far more urea than could have been present at any time during hydrolyses of *d*-arginine in the presence of the jack bean enzymes under the conditions described (see (9)).

Procedure — Solutions of *d*-arginine hydrochloride and jack bean preparation with cobaltous ion and buffer were mixed and kept at 30.0°. The time of mixing was noted and at definite intervals (*t* in Fig. 2) a 20 ml. sample was withdrawn from the reaction mixture. This was added to 1 ml. of hydrochloric acid

(1 M) in a 25 ml. volumetric flask, kept at 100° for 10 minutes and cooled. After the addition of water to the mark and subsequent filtration, 10 ml. samples were removed and aerated in the usual



t	h	s	k
hours	ml. 0.020 N HCl		inverse hours
0	0.00	9.50	——
0.5	0.59	8.91	0.128
1	1.24	8.26	0.140
2	2.33	7.17	0.141
3	3.23	6.27	0.139
4	4.03	5.47	0.138
5	4.72	4.78	0.138
23	8.71	0.79	(0.124)

FIG. 2. *d*-Arginine hydrolysis in the presence of jack bean arginase and cobaltous ion. The reaction mixture, total volume 160 ml., contained *d*-arginine hydrochloride 0.400 gm., $CoCl_2 \cdot 6H_2O$ 0.016 gm., phosphate buffer 1 M (pH 7.5) 80 ml., crude crystalline jack bean urease 8 ml., redistilled water to volume, a few drops of caprylic alcohol. The enzyme solution (8 ml.) contained crude crystalline urease derived from 32 gm. of jack bean meal (of an Arkansas crop purchased on the market). The rate of disappearance of the substrate under the conditions may be formulated as $-ds/dt = ks$, from which $k = (2.303/t) \times$ (log a minus log s), where k = the "specific reaction" constant, s = moles of substrate (*d*-arginine) present at time t, and a = the initial *d*-arginine concentration. For a may be used the calculated ml. of 0.0200 N HCl equivalent to the ammonia corresponding to complete hydrolysis of the *d*-arginine in 0.4 of a 20 ml. sample of reaction mixture (see the text); on the basis of 2 equivalents of NH_3, a = 9.50. For s is used the value obtained by subtracting from a the ml. of 0.0200 N HCl required to neutralize the NH_3 formed at time t (h column). (The values listed for h have been corrected for the control on reagents and enzyme in absence of substrate; control = 0.10 ml. of 0.0200 N HCl.)

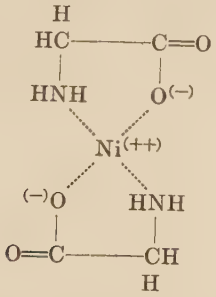
manner for the estimation of ammonia. Further details and the results are given in Fig. 2.

Potentiometric Titrations; Distortion of Titration Curve of d-

Arginine in Presence of Ni^{++}, Co^{++}, or Mn^{++} — Data presented in this and the following paper disclose that effective heavy metal ions not only "activate" the argininolytic enzymes, but, in addition, induce shifts in their activity-pH curves. As a preliminary, it seemed appropriate to inquire to what extent the modifications are related to the ability of the substrate, *d*-arginine, to form complexes with such ions. An exploratory study has been made with cobaltous, nickelous, and manganous salts.

From the introductory paragraphs it might be inferred that an extension of the range of enzyme activity into a more acid region in the presence of cobaltous ion, for example, is to be correlated with the ability of cobaltous (or more pertinently the enzyme-cobaltous) ion to compete effectively in that region with hydrogen ion for a donor group such as the α-amino group. In addition, the precipitation of a metallous hydroxide would play its own part in the modification of the activity-pH relations.

In our experience the actual isolation of nickelous or cobaltous complexes of arginine is not so simple a matter as the preparation of corresponding complexes derived from glycine or alanine. The latter were first studied by Ley (12). Nickelous glycine, neutral in character, may be designated by the accompanying formula. Although the situation is probably somewhat similar



for *d*-arginine, the presence of guanidinium groupings may influence the physical properties of the complexes (here, complex ions) in the direction of increased solubility, etc.

Early observation that complex formation with amino acids hinders the alkaline precipitation of metal ions has been elaborated by so called anomolous titrations. Such titrations have been

made upon mixtures of glycine and Mg^{++} by Zörkendörfer (13), upon hydroxy acids and Fe^{+++} or Cu^{++} by Smythe (14), and upon hydroxy acids (and arginine) and Mn^{++} by Main and Schmidt (15). In these studies there have been disclosed interesting shifts in the titration curves of various compounds in the presence of metal ions.

In order to obtain data which might permit an approximation of the order of magnitude of the dissociation constants of metallo-arginine complexes, there have been carried out in this work a number of potentiometric titrations. With sodium hydroxide, solutions of nickelous, cobaltous, and manganous chlorides were titrated for the determination of the ranges of pH in which the ions are precipitated as "hydroxides" (*cf.* (16, 17)). In addition, *d*-arginine hydrochloride has been titrated in the presence of each of these salts. To the metal salt solutions to be titrated was added sodium chloride to its concentration in the arginine-metal ion titration mixtures at the point of hydroxide precipitation (*i.e.*, about equivalent to the sodium hydroxide used). In the zones of precipitation there were encountered drifts in the hydrogen ion activity to more acid values, which in some cases persisted for several hours before the change in pH during 10 minutes was 0.01 pH unit or less; therefore, care was taken to determine carefully several points after the first definite precipitation of a metallous hydroxide was observed. Titrations were attempted at different times with both glass and hydrogen electrodes, the working half-cell being a saturated calomel electrode. The glass electrode used permitted the measurement of changes in pH of 0.005 pH unit. It was calibrated on each day of use by measurement of six or seven appropriate buffers, the pH of which had been determined with a hydrogen electrode. In the range of pH 2.0 to 8.0, there were obtained with the glass electrode the theoretical values of $\Delta E/\Delta \mathrm{pH}$; for the more alkaline range there were used calibration data in order to convert measured potentials to pH units. For the measurements with glass electrodes, the temperature was controlled in an air bath at $30.0° \pm 0.03°$. Purified nitrogen was used to stir the solutions and exclude air from the titration vessel, which was similar to that described by Clark (16).

Discrepancies were noted in the determination of the pH zones of precipitation of the metal hydroxides both in the presence and

absence of arginine when the results obtained respectively with the glass and hydrogen electrodes were compared. The values obtained with the glass electrode are considered more reliable, for apparently the glass electrode is not affected by the hydroxides nor are the latter altered by the electrode. Nevertheless, the general results with both electrodes are similar qualitatively. So far as titrations of amino acid alone are concerned, they agree quantitatively. The heavy metal salts used were high grade commercial products; they were analyzed and upon the analyses were based the concentrations of metal ions used in the titrations. By precipitation of nickelous dimethylglyoxime, the $NiCl_2 \cdot 6H_2O$ used was found to contain the theoretical amount of nickel. The $CoCl_2 \cdot 6H_2O$ was found, by potentiometric titration with potassium ferricyanide (18), to contain 97.1 per cent of the theoretical cobalt content. In the $MnCl_2 \cdot 4H_2O$, the manganese content was found by the use of sodium bismuthate to be 99.6 per cent of the theoretical amount. The purity of *d*-arginine and other organic compounds titrated was established by suitable analyses (total and α-amino N) and by the correspondence of the theoretical and observed end-points in appropriate titrations.

In addition to *d*-arginine, a number of other compounds were titrated in the presence and in the absence of Co^{++}, Ni^{++}, and Mn^{++}. Such compounds were glycine, δ-guanidinovaleric acid, and *d*-ornithine dihydrochloride. In most of these cases, the detailed characteristics are of little concern to the central topic of the present communications and are not presented here. They will be considered as occasion demands.

Fig. 3 presents the titration curves (with the glass electrode) of *d*-arginine in the presence of and in the absence of several heavy metal salts. With the detail of the foregoing text the figure is self-explanatory. By way of illustration of the titrations of metal salts alone some data are presented from the titrations of nickelous chloride in the absence of amino acid. Table I gives illustrative results.

An analysis of the potentiometric titrations might permit some decisions regarding both the composition of the *d*-arginine-metallic

complexes and the dissociation constants thereof. However, there would necessarily have to enter certain assumptions, the verification of which would require more extensive investigation. Since the immediate objective is met by a semiquantitative analysis of the data at hand, we prefer to leave the more rigid analysis to the future and shall proceed with the argument within restricted premises. On the basis of available collateral information concerning the nature of such nickelous complexes of the simpler amino acids as have been isolated (12) and of the nickelous, cobaltous, and manganous ammonium ions, it might be supposed that arginine forms with such metal ions an equilibrium mixture of complexes in which the form 2 arginine-1 metal ion quantitatively is an important component. Borsook and Thimann (19) came to a similar conclusion in their studies of cupric glycine. If it be assumed that a complex ion of this type is most characteristic for a divalent metal ion-arginine complex *in the pH region where precipitation of the metal hydroxide is imminent*, the dissociation constant K_c of the postulated complex $((RNH_2)_2Me)^{++}$ may be calculated with the aid of the expression

$$\frac{[Me^{++}][RNH_2]^2}{[((RNH_2)_2Me)^{++}]} = K_c$$

Substitutions are made with the use of the solubility product relation $K_s = [Me^{++}][OH^-]^2$ for a given metal hydroxide and the equilibrium relation for the dissociation of arginine's α-ammonium group (K'_2 under the conditions (see text) is taken as 1.29×10^{-9}). Illustrative results for such calculations, based upon the data obtained in this work, are as follows:[2]

Metal hydroxide	K_s	Postulated complex	K_c
Nickelous.....	4.5×10^{-15}	$(Ni\ (arginine)_2)^{++}$	6×10^{-11}
Cobaltous.....	6.5×10^{-15}	$(Co\ (arginine)_2)^{++}$	2×10^{-8}
Manganous.....	1.3×10^{-13}	$(Mn\ (arginine)_2)^{++}$	7×10^{-5}

[2] Approximations of the dissociation constants of nickelous-, cobaltous-, and manganous-arginine, presented in a preliminary report (20), were based upon data obtained with the use of the hydrogen electrode. For reasons already cited, they are considered less reliable than the values given here.

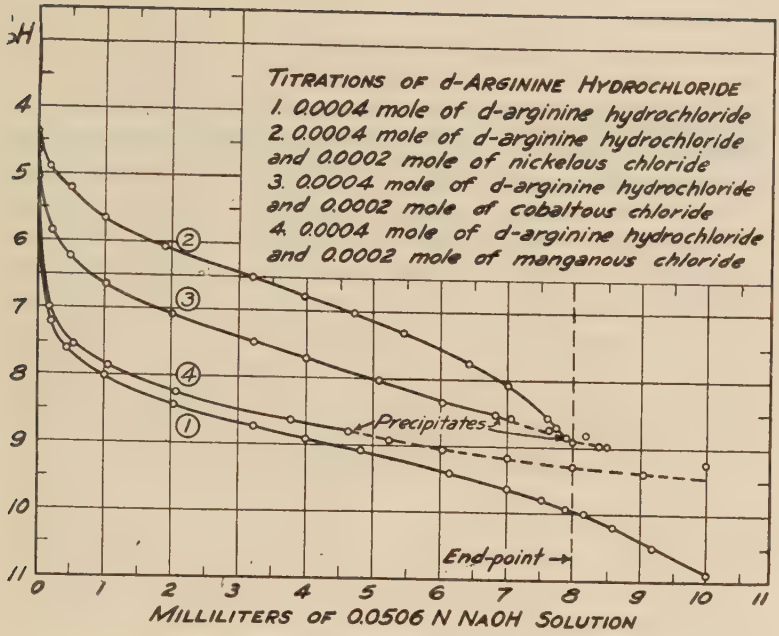

pH
TITRATIONS OF d-ARGININE HYDROCHLORIDE
1. 0.0004 mole of d-arginine hydrochloride
2. 0.0004 mole of d-arginine hydrochloride and 0.0002 mole of nickelous chloride
3. 0.0004 mole of d-arginine hydrochloride and 0.0002 mole of cobaltous chloride
4. 0.0004 mole of d-arginine hydrochloride and 0.0002 mole of manganous chloride
Precipitates
End-point
MILLILITERS OF 0.0506 N NaOH SOLUTION

FIG. 3

TABLE I

Titrations of Nickelous Chloride with Sodium Hydroxide

Solution A. 40 ml. containing 0.0002 mole of nickelous chloride and 0.00032 mole of sodium chloride. (The latter salt was added in an amount equal to that formed during the titration of arginine hydrochloride and nickelous chloride to the point of precipitation of nickelous hydroxide.)

Solution B. 50.4 ml. containing 0.0002 mole of nickelous chloride and 0.0004 mole of sodium chloride.

A glass electrode was used; temperature, 30.0° ± 0.03°.

Solution A		Solution B	
0.0506 N NaOH	pH	0.0500 N NaOH	pH
ml.		*ml.*	
0.00	6.20	0.00	5.45
0.04	7.82	0.03	6.45
0.08	7.90*	0.06	6.89
0.12	7.93†	0.17	7.90*
	7.75‡	0.23	7.97
		0.45	8.00
0.82	7.92†	0.75	8.00
	7.75‡	0.97	8.00

* Precipitation of metal hydroxide.

† Initial pH after addition of NaOH.

‡ pH at which drifts ceased.

The determination of the hydrogen ion concentration at the point where a metal hydroxide just begins to precipitate when an amino acid-heavy metal salt mixture is titrated with sodium hydroxide rests upon the assumption that the solutions are in true equilibrium with the solid phase which, for present purposes, is considered to consist essentially of the species $Me(OH)_2$. This introduces a rather important source of uncertainty. The observations upon which the illustrative results are based were found to be fairly reproducible. It may be noted that an error in the determination of pH at the precipitation point amounting to 0.05 to 0.1 pH unit would change the values of K_c within a range of 1×10^1. The experimental difficulties and the assumptions underlying the calculations of the dissociation constants, K_c, strictly limit their applicability. However, the numerical values, presented for comparative purposes, suffice as tentative approximations. A more rigorous analysis should furnish a more general and useful evaluation.

DISCUSSION

In Fig. 1 is depicted the effect of certain activating heavy metal ions upon *d*-arginine hydrolysis in the presence of the jack bean argininolytic enzyme with buffers of widely varying pH values but of approximately constant ionic strength. Further elaboration of such data did not seem advisable with the enzyme preparations available. The activity-pH curves show characteristic differences. Manifest is the enhancement of catalytic activity in the presence of cobaltous and of manganous ion. Of particular interest is the ability of the cobaltous ion to effect an extension of the range of enzyme activity into a pH region considerably less alkaline than that observed for the enzyme without added heavy metal salt. The range of optimal activity is shifted from the region of pH 9 to about pH 7.7. Even more striking is the steep ascent to its first inflection of the curve representing cobaltous-activated enzyme; the rise to a maximum is within a pH unit or less. This is in marked contrast to the behavior of liver arginase (see Fig. 1 and (11)). For the latter enzyme the inception of cobaltous activation is at pH 4.5, the activity being maximal at pH 7.5. On the other hand, jack bean enzyme with Co^{++} displays negligible activity below pH 6.7, and maximal at pH 7.7. Another

point of difference is seen in the behavior of the enzymes with nickelous ion; with nickel salts, under the conditions used, the jack bean enzyme is not efficiently activated. These and other differences constitute strong evidence of the non-identity of the two catalysts as chemical individuals.

From the observations of negligible jack bean argininolytic activity below pH 6.7 (with or without added metal ion) arises a consideration of some importance in analytical work. For example, an adjustment of the hydrogen ion concentration to pH 6.0 in a reaction mixture containing jack bean enzymes can be used to eliminate the argininolytic activity; under the conditions the ureolytic principle remains sufficiently active for analytical purposes.

The activity-pH curve for cobaltous-enzyme displays an *apparent* subsidiary optimum around pH 9, which is approximately the region of characteristic inflection for the "native" jack bean enzyme. There is a *suggestion* of a second optimum also in the curve for manganous-enzyme. Elaboration of the argument that each of these curves reflects the properties of a mixture of enzymes differing only in their metallic components would be gratuitous at present. Similar behavior is exhibited by liver arginase (11).

Although it has been found convenient to visualize the argininolytic enzymes as metallic complexes and this conception has aided experimentation, it must be recalled that there is as yet little direct evidence of their chemical nature. Moreover, there is no anticipation that the structure of any coordination complex elaborated by combination of a metallo-enzyme and its substrate, or the stability of the resulting molecule and its other attributes, would be closely comparable with the corresponding characteristics of a simple amino acid-metal complex such as nickelous-arginine or nickelous-glycine. Nevertheless, it was deemed advisable, for reasons discussed earlier in this paper and in order to gain additional information regarding the general behavior with amino acids of the activating ions, to ascertain the relative abilities of certain of these ions to combine with *d*-arginine. The measurements have shown that for the salts used specifically in the activity-pH studies, the respective ions in the order of stability of their complexes with *d*-arginine are $Ni^{++} > Co^{++} > Mn^{++}$. A similar relationship also was found to obtain, qualitatively,

for the corresponding complexes of glycine. The presence of heavy metal ions used in these titrations has thus the effect of displacing the titration curves of the α-amino groups of either glycine or *d*-arginine to a more acid region. In titrations designed to indicate the shift in pH with respect to variation in the ratio of concentrations of cobaltous (or nickelous) ion to *d*-arginine, it was observed that the greater shifts occurred when a comparatively small proportion of metal ion had been added; for example, there was little further shift in the titration curve of *d*-arginine's α-amino group when there had been added heavy metal ion in excess of an amount equivalent to the *d*-arginine present. While these titrations have yielded no final information concerning the composition of the complexes, they are of interest in connection with some observations of Levy (21) dealing with titrations of *d*-arginine in the presence of formaldehyde. Of the latter reagent, an amount greatly in excess of the concentration of *d*-arginine was required to produce the largest shifts in the titration curves.

Of considerable importance to this investigation is the question whether a relationship exists in the variations to be noted in the activity-pH curves and the relative ability of effective activating ions, in general, to form coordination compounds; *e.g.*, with *d*-arginine. In considering this question it must be emphasized that the conditions under which the two sets of data were obtained are conspicuously different. For example, heavy buffering is, of necessity, required in the pH-activity studies. Furthermore, the postulated non-metallic donor component[3] of the enzyme would play a rôle in the shaping of the pH-activity curves, not alone through variation of its stability with changing pH (*cf.* (22)) but further through changes in ionic species involving groups more or less associated with its activity. Such effects may also account in part for the major differences in the corresponding pH-activity curves for liver arginase and the jack bean enzyme. The ability of effective heavy metal ions to cause a shift in the enzyme's pH-activity curve is less apparent in jack bean arginase than in liver arginase (11), because the pH range of activity is in general less broad in the former case. Nevertheless, it seems clear that

[3] *Metallo-enzyme* would represent a kind of "holoenzyme"; *donor component* of arginase, the corresponding "apoenzyme" (*cf.* (23)).

under the conditions cobaltous ion has widened the range of activity of the jack bean enzyme also, effecting a definite *shift* of the ascending branch of the activity curve into a less alkaline region. A similar extension evidently is not associated with the action of the manganous ion, an almost equally potent activator. It is tempting to correlate these observations with the findings concerning the stability of *d*-arginine-metallic complexes; specifically, that the stability of certain *d*-arginine-cobaltous complexes is appreciably greater than of supposedly comparable *d*-arginine-manganous complexes. Is the shift of the activity curve in the presence of the cobaltous ion to be attributed, in a measure, to the ability of the metal ion, through coordination with α-amino groups (and carboxyl anions) to "withdraw" the substrate, *d*-arginine, in the region of activity of the corresponding metallo-enzyme?

There remain to be considered the results of experiments dealing with the kinetics of hydrolysis of *d*-arginine. When the catalyst is jack bean arginase with a cobaltous salt, the process, under the conditions, is pseudomonomolecular. This is comparable with our previous observations with liver arginase. However, in the present instance urea, a product of the initial hydrolysis, does not accumulate, since urease is a constituent of the enzyme preparation used. In an earlier report ((1) p. 189) the question was raised whether the argininolytic effect here might be attributable essentially to the efficient removal of the urea formed in an initial hydrolysis of a metal-activated complex of *d*-arginine. A consideration of the relative rates of hydrolysis in the involved reactions has permitted a negative answer. In addition, as will be shown elsewhere, the *non-enzymatic* (hydroxyl ion-catalyzed) hydrolysis of *d*-arginine to urea and ornithine is very slow and is not hastened in appropriate regions of pH by the presence of small amounts of metal ions which can activate the argininolytic enzymes. The results from rate measurements are consistent with the observations referred to in the introductory section respecting the negligible effect upon *d*-arginine of cobaltous salts with highly recrystallized urease. The argininolytic action is evidently independent of the ureolytic.

In general, the results of this investigation, while suggesting

the non-identity of liver arginase and the jack bean argininolytic enzyme,[4] at the same time emphasize a similarity in the mode of their participation in *d*-arginine hydrolysis. For the two enzymes there has been indicated also a similar specificity (11).

SUMMARY

1. For exploratory purposes there have been evaluated under a severely restricted set of conditions activity-pH curves for the jack bean argininolytic enzyme. These curves depict characteristic changes induced by several effective activating heavy metal ions. The data have suggested a useful application in the estimation of urea in the presence of *d*-arginine and in the evaluation of liver arginase activity.

2. By means of appropriate potentiometric titrations there was ascertained the relative stability of complexes formed by *d*-arginine with nickelous, cobaltous, and manganous ions. The bearing of the data upon certain characteristics of the pH-activity curves has been considered.

3. Studies of the kinetics of *d*-arginine hydrolysis in the presence of the jack bean enzyme and cobaltous ion at pH 7.5 have disclosed that the process, similar to hydrolysis catalyzed by liver arginase, is pseudomonomolecular. The argininolytic activity of the jack bean depends in no observable way upon urease as such.

4. There have been pointed out certain differences in the characteristics of liver arginase and the jack bean enzyme as well as the apparent similarity in the mode of their "activation" and of their action. The results appear to have significance for the elucidation of the rôle of metal ions in the enzymatic hydrolysis of *d*-arginine.

[4] Iwabuchi has reported (24) failure to confirm the argininolytic action of jack bean preparations. Since he does not present his data, his work cannot be evaluated. His experiments may have been conducted in ranges of pH unfavorable to the enzyme's activity or his preparations may have been poor in the argininolytic factor. Sumner and Dounce (2) have confirmed the observations (1) relative to the arginase-like property of certain jack bean preparations with cobalt salts. It is important to note that whole jack bean meal contains a hydrolyzable substrate upon which an enzyme, also contained in the meal, may act under suitable conditions, especially in the presence of activating heavy metal salts. It is conceivable that the substrate is canavanine and that the canavanase is identical with the enzyme, termed in this paper, "jack bean arginase" (*cf.* (11)).

BIBLIOGRAPHY

1. Hellerman, L., and Perkins, M. E., *J. Biol. Chem.*, **112**, 175 (1935–36).
2. Sumner, J. B., and Dounce, A. L., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **119**, p. xcvii (1937).
3. Hellerman, L., *Physiol. Rev.*, **17**, 454 (1937).
4. Cohn, E. J., *Ergebn. Physiol.*, **33**, 824 (1931).
5. Hunter, A., and Morrell, J. A., *Tr. Roy. Soc. Canada*, **16**, sect. 5, 75 (1922); *J. Soc. Chem. Ind.*, **43**, 691 (1924); *Quart. J. Exp. Physiol.*, **23**, 89 (1933).
6. Edlbacher, S., and Bonem, P., *Z. physiol. Chem.*, **145**, 69 (1925).
7. Edlbacher, S., and Simons, E., *Z. physiol. Chem.*, **167**, 76 (1927).
8. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 211 (1914).
9. Sumner, J. B., *J. Biol. Chem.*, **69**, 435 (1926); also in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipsic, **1**, 295 (1932).
10. Green, A. A., *J. Am. Chem. Soc.*, **55**, 2331 (1933).
11. Hellerman, L., and Stock, C. C., *J. Biol. Chem.*, **125**, 771 (1938).
12. Ley, H., *Ber. chem. Ges.*, **42**, 3894 (1909); **45**, 372 (1912); *Z. Elektrochem.*, **10**, 954 (1904).
13. Zörkendörfer, W., *Biochem. Z.*, **221**, 33 (1930).
14. Smythe, C. V., *J. Biol. Chem.*, **92**, 233 (1931).
15. Main, R. K., and Schmidt, C. L. A., *J. Gen. Physiol.*, **19**, 127 (1935).
16. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition (1928).
17. Britton, H. T. S., *J. Chem. Soc.*, **127**, 2110, 2148 (1925).
18. Tomicek, O., and Freiberger, F., *J. Am. Chem. Soc.*, **57**, 801 (1935).
19. Borsook, H., and Thimann, K. V., *J. Biol. Chem.*, **98**, 671 (1932).
20. Hellerman, L., and Stock, C. C., *J. Am. Chem. Soc.*, **58**, 2654 (1936).
21. Levy, M., *J. Biol. Chem.*, **99**, 767 (1932–33); **109**, 365 (1935).
22. Hunter, A., and Dauphinee, J. A., *J. Biol. Chem.*, **85**, 627 (1929–30); *Quart. J. Exp. Physiol.*, **23**, 119 (1933).
23. Albers, H., *Angew. Chem.*, **49**, 448 (1936).
24. Iwabuchi, T., *J. Biochem.*, Japan, **24**, 447 (1936).

ACTIVATION OF ENZYMES*

V. THE SPECIFICITY OF ARGINASE AND THE NON-ENZYMATIC HYDROLYSIS OF GUANIDINO COMPOUNDS. ACTIVATING METAL IONS AND LIVER ARGINASE

By C. CHESTER STOCK

(From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore)

(Received for publication, July 16, 1938)

More recent studies of the properties of arginase have stressed the probable significance of metal ion chemistry for the mechanism of its action (1). Previous reports (2, 3) from this laboratory have dealt with the inhibition of activity of liver arginase by a variety of reagents and the restoration of activity by certain heavy metal ions (especially Co^{++}, Ni^{++}, and Mn^{++}), with the existence of an argininolytic enzyme in the jack bean which also is remarkably activated in the presence of certain of these ions, and with evidence pointing to a possible rôle of metal-coordinative processes in the enzymatic hydrolysis of *d*-arginine.

From potentiometric titrations of *d*-arginine in the presence of heavy metal ions (3) it is clear that the α-amino group plays an important rôle in the formation of metallo complexes derived from this amino acid. In this respect arginine as compared with simpler amino acids (*e.g.*, glycine) displays no essential peculiarity. This raises a question with reference to the necessity for the α-amino substituent in the substrate upon which the argininolytic enzymes act. Insufficient information upon this point has been afforded by the already extensive studies upon the specificity of arginase.

In this paper are recorded observations which depict the modifications induced in the activity-pH curve of liver arginase under

* For related studies dealing with certain adjacent fields of biological catalysis and not included in the numbered series, see (1) and *Am. J. Physiol.*, **120**, 522 (1937).

certain conditions in the presence of manganous, cobaltous, or nickelous salts. Circumstantial evidence suggesting that the α-amino group of *d*-arginine (*S*) may play a rôle in "anchoring" the postulated metallo-enzyme (*E*) in the sense of Equation 1

$$E + S \rightleftarrows ES \qquad (1)$$

is strengthened by more direct evidence bearing upon the indispensability of this substituent group for the enzyme's *efficient* functioning. It is shown that the hydrolysis of neither δ-guanidinovaleric acid nor argininic (δ-guanidino-α-hydroxyvaleric) acid is effectively catalyzed by liver or jack bean arginase. The results reinforce the observations of other investigators in emphasizing the rather striking specificity in the enzymatic hydrolysis of *d*-arginine. This has prompted a comparison with the kinetics of hydrolysis of various guanidino compounds in the presence of an adequate concentration of hydroxyl ion to replace the enzymes as catalysts. Conditions are described for the controlled hydrolysis of *d*-arginine, δ-guanidinovaleric acid, argininic acid, glycocyamine, and guanidine. There have appeared important distinctions in the characteristics of the enzymatic and hydroxyl ion-catalyzed processes; in addition, the results have disclosed that *d*-arginine, as compared with other guanidines, possesses no unusual inherent instability toward hydrolysis.

Current studies in this laboratory are emphasizing also the nature of arginase as it occurs in tissue extracts.

EXPERIMENTAL

Activity-pH Curves of Liver Arginase; Effect of Certain Heavy Metal Salts—The preparation of crude arginase was carried out according to the directions of Hellerman and Perkins (2). It was found most convenient to reextract calf liver tissue several times and to combine the fluid extracts before going on with the several manipulations preceding the acetone precipitation. The use of glycerol is probably unnecessary. The use of a centrifuge before the precipitation step ordinarily may be omitted. Ample acetone is required in the drying process. From the dried acetone precipitate, *arginase solution* was prepared as previously directed.

The activity-pH curves were evaluated on the same basis as that described by us previously (3). It was considered unneces-

sary at this time to try to employ a more ideal procedure. There is considerable variation in the rate of destruction of liver arginase with change in the hydrogen ion concentration in certain regions (4). With a reasonably "stable" enzyme preparation and the use of relatively short periods of hydrolysis, there were obtained data with which could be delineated the major differences in the activity curves.

The buffers used, the pH intervals at which observations were taken, the ionic strength in *d*-arginine-arginase mixtures, and the amounts of activating heavy metal salts added were all as specified in the preceding paper (3) in which we presented activity-pH curves of jack bean arginase.

The arginase solutions used varied somewhat in enzyme content. It was impracticable to make from one preparation simultaneous determinations of all the necessary data. However, in repeated determinations the zones of pH for optimal activity in the case of each enzyme preparation remained the same. In addition, the relative degree of activation referable to each of the several added metal ions was substantially as given in Fig. 1, which presents mean curves based on all the data.

Procedure—Water redistilled in glass was used. The reaction vessels were 25 ml. volumetric flasks of "resistance" glass. To a solution containing 10 ml. of buffer, 46 mg. of *d*-arginine hydrochloride, and appropriate heavy metal salt were added 5 ml. of arginase solution and water to make a total volume of 20 ml. After the further addition of 2 drops of caprylic alcohol, the mixture was kept at 37° for 3½ hours and then heated at 100° for 10 minutes (to destroy arginase). Adjustment to pH 7 was made and there were added 16 mg. of sodium cyanide, 0.1 gm. of crude urease, and 1 M phosphate buffer of pH 7.5 to the 25 ml. mark. A little caprylic alcohol was added and the mixture was permitted to stand at 30° for about 16 hours, after which it was filtered and the ammonia in 10 ml. of the filtrate estimated in the usual manner (2) in terms of ml. of 0.0200 N HCl neutralized.

Elaborate controls were carried out upon reagents and heat-denatured enzyme; the controls included determinations in which substrate or enzyme was omitted and estimations of pure urea under the conditions of the various runs. There were obtained blanks representing ammonia from the enzyme and reagents

equivalent to as much as 0.35 ml. of 0.0200 N HCl. Suitable corrections were applied throughout.

Note on Estimation of Urea—As pointed out by Stock, Perkins, and Hellerman, the activity-pH curves for the jack bean arginin-olytic enzyme suggest that the "arginase activity" of jack bean preparations, even in the presence of the activating ions, Co^{++}, Mn^{++}, or Ni^{++}, may be abolished without gross impairment of the ureolytic activity if the hydrogen ion concentration of a reaction

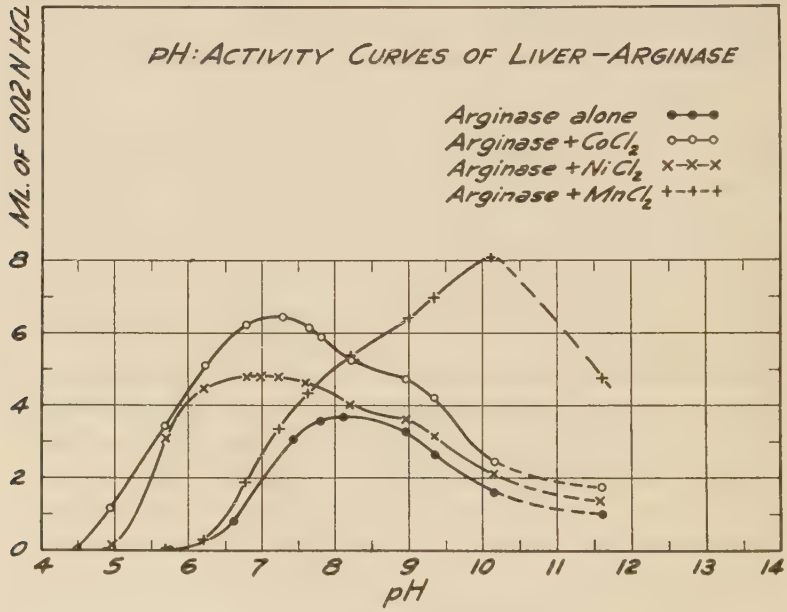


Fig. 1

mixture is adjusted to a value below pH 6.5. Dr. M. M. Richards finds[1] that liver arginase activity may be reliably evaluated or urea in the presence of *d*-arginine and "activating" salts accurately determined under conditions similar to those of the "Procedure" if the reaction mixture (after being heated and cooled) is treated with 16 mg. of sodium cyanide and then brought to pH 6 by the addition of hydrochloric acid before the addition of urease and buffer solution of pH 6. The use of cyanide is still found advantageous (2); hydrocyanic acid supports the action of urease (1). After

[1] Unpublished experiments.

2 hours at 30°, the reaction mixture is analyzed for ammonia by 2 hours aeration in the usual fashion. The titration mixture may be stirred with a stream of clean, CO_2-free air, chlor-phenol red being a convenient indicator.

Specificity of Arginase in Relation to Rôle of α-Amino Group in d-Arginine—The work of a number of investigators has suggested that alterations in the *d*-arginine molecule (*e.g.*, esterification) prevent or markedly hinder the action of arginase. Inactivity has been observed with certain lower analogues and with some α-amino-substituted derivatives. In addition, the specificity concerned with the enantiomorphs of arginine is at least one of degree (5). Information concerning the essentialness of the α-amino group is conflicting or indecisive. It has been reported that one guanidino group of arginylarginine is hydrolyzed (6), that α, N-benzoylarginine is hydrolyzed at pH 6.5 to 7.5 (but not above or below this pH range) (7), and that α,N-methylarginine is not attacked (8). Observations of argininic acid hydrolysis in the presence of arginase were reported by Calvery and Block (9) and by Felix and Muller (10), although Felix, Muller, and Dirr (7) had failed previously to observe such hydrolysis.

The present investigation includes a study of the behavior of our preparations of liver arginase and of jack bean arginase, in the absence and in the presence of certain activating metal salts, with certain analogues of arginine (δ-guanidinovaleric acid and δ-guanidino-α-hydroxyvaleric (argininic) acid) *as compared with* the behavior of these enzymes with *d*-arginine under the same conditions.

Preparation of δ-Guanidinovaleric Acid—The method of preparation is that of Ackermann, Engeland, and Kutscher (11). They reported that an abundant crust of the compound is obtained after an ammoniacal solution containing specified amounts of cyanamide and δ-aminovaleric acid is allowed to stand for 5 weeks. The product was analyzed (N). According to conventional formulas, the process is depicted thus:

$$H_2N{-}C{\equiv}N + H_2N{-}(CH_2)_4{-}COOH \rightarrow H_2N{-}\overset{\overset{NH}{\|}}{C}{-}\overset{\overset{H}{|}}{N}{-}(CH_2)_4{-}COOH$$

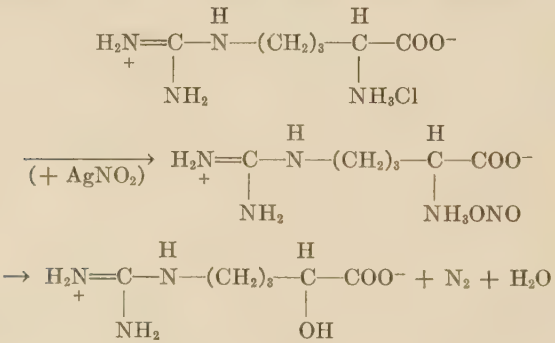
We proceeded as follows: δ-Aminovaleric acid hydrochloride, 5.06 gm., and cyanamide, 1.41 gm., were dissolved in the minimum amount of water in a 50 ml. beaker. The solution was made

slightly alkaline with ammonia. After 3 weeks, hard clusters of crystals of δ-guanidinovaleric acid had formed; these were collected, washed with 95 per cent ethanol, and crystallized from hot water. The solution gave a positive Sakaguchi test (12) and a negative chloride test. The product decomposed at 273°; it was ground with absolute ethanol, freed of alcohol by filtration, and recrystallized from hot water. Two preparations yielded 4.62 gm. The capillary "melting point" was observed in a bath preheated to 270°; decomposition point, 283.5–284.5° (corrected). The previously reported melting point was 265–266°.

	C	H	N	Ash
Calculated for $C_6H_{13}N_3O_2$.....	45.25	8.2	26.4	
Found (Pregl microcombustions)*..	45.26	8.1	26.5	0.12

Sodium hydroxide may be used instead of ammonia to bring the reaction mixtures to slight alkalinity. From preparations in which were used 3.0 gm. of δ-aminovaleric acid hydrochloride and an equivalent amount of cyanamide with sodium hydroxide there were obtained in 2 days yields of 1.0 to 1.5 gm. of δ-guanidinovaleric acid. The products contained little ash.

Preparation of Argininic Acid—The method of Felix and Muller (13) was used. Some deviation was found necessary, owing to



the sluggishness of the reactions which involved the splitting of nitrogen (as N_2) from arginine nitrite, and this has since been noted also by Felix and Muller (10). The method of preparation may be illustrated by the accompanying scheme.

A solution of 6.96 gm. of *d*-arginine hydrochloride and 5.57

* For the microcombustions we are indebted to Dr. R. T. Milner and Mrs. H. S. Sherman of Washington, D. C.

gm. of silver nitrite in 350 ml. of water was stirred mechanically for an hour. The precipitated silver chloride was removed by filtration. The filtrate, which was found by test to be chloride-free, was saturated with hydrogen sulfide to remove remaining silver ions. After the removal of silver sulfide, the solution was concentrated under reduced pressure to about 50 ml. Ethanol was added until the mixture was slightly turbid; from the cooled mixture there were obtained 2.16 gm. of crystalline arginine nitrite. When ether was added to the mother liquor, there was obtained a second crop of 3.64 gm. Samples from both crops decomposed at 172–174°. The previously reported decomposition point was 160°. The theoretical yield was 7.3 gm. as compared with 5.8 gm. obtained. The product gave a positive test for nitrite with acidified potassium iodide. As a nitrite it also was found to reduce approximately the calculated amount of potassium permanganate.

Of arginine nitrite, 4.3 gm. were utilized for the preparation of argininic acid. The nitrite was found unusually resistant to decomposition when heated in an aqueous solution. It was found necessary to heat it in a concentrated aqueous solution with the use of a glycerol bath held at 120 130° for 5 to 8 hours; addition during this period of 50 ml. of 0.1 M HCl was also necessary to insure completion of the action. After the heating, the solution was evaporated to dryness under reduced pressure. The residual solid was taken up in the minimum amount of warm water, and the solution was treated with absolute ethanol and chilled. The yield of crystallized product was 1.17 gm. and of a less pure residue in addition, 0.75 gm. A solution of a sample of this argininic acid had a pH value of about 7.8. Tests for chloride and nitrite ion were negative.

This method for the preparation of δ-guanidino-α-hydroxyvaleric acid is not regarded by us as entirely satisfactory or as fully conclusive of the structure of the product. A method of synthesis would be preferable in which is avoided the decomposition (14) of an amine nitrite grouping in a molecule having a guanidino substituent. However, estimations by the Van Slyke procedure indicated the presence in the product of 0.42 per cent or less of α-amino nitrogen; the total nitrogen found (micro-Dumas) was 23.6 per cent as compared with 23.9 per cent cal-

culated. The product had in general the properties described by Felix and Muller. It was found also that the alkali-catalyzed hydrolysis of the compound yielded urea and ammonia and was comparable in rate with that of a number of other guanidino compounds. This is detailed in a later section.

For present purposes the configuration of the α-carbon atom

TABLE I

Liver Arginase with δ-Guanidinovaleric Acid and with Argininic Acid

Substrates are as follows: *d*-arginine hydrochloride, δ-guanidinovaleric acid, and argininic acid, in amounts designated; the enzyme is liver arginase solution (described earlier in the paper and in (2)), 2 ml.; buffer in Experiments 1 to 3, 5 ml. of phosphate (1 M, pH 7.45) and in Experiments 4 to 6, 5 ml. of glycine (constituents as in Sørensen's, 1 M, pH 9.5); redistilled (in glass) water to a total volume of 20 ml.; caprylic alcohol, 2 drops; digestion, 20 hours at 37°; urea in two-fifths of the reaction mixture estimated as usual (see an earlier section). The results are expressed as ml. of 0.02 N HCl equivalent to ammonia from the urea formed and hydrolyzed.

Experiment No.	pH	Substrate*		0.02 N HCl neutralized	Substrate hydrolyzed
			mg.	*ml.*	*per cent*
1	7.45	*d*-Arginine hydrochloride	42	5.30	66
2	7.45	δ-Guanidinovaleric acid	32	0.00	0
3	7.45	Argininic acid†	35	0.44	0
4	9.5	*d*-Arginine hydrochloride	42	7.32	92
5	9.5	δ-Guanidinovaleric acid	32	0.00	0
6	9.5	Argininic acid†	35	0.44	0

* Appropriate controls were made throughout. When substrate was absent, or enzyme was heated at 100° before addition, the ml. of HCl were 0.17 to 0.20. The values given have been corrected with these blanks. Total hydrolysis of two-fifths of the arginine hydrochloride used would produce urea ammonia equivalent to 8.00 ml. of 0.02 N HCl; of the δ-guanidinovaleric acid, 7.83 ml.; of the argininic acid, 8.00 ml.

† Determination of α-amino N (Van Slyke) indicated that the argininic acid used contained sufficient *d*-arginine to yield ammonia equivalent to 0.42 ml. of 0.02 N HCl.

with respect to asymmetry was neglected. (The starting material for argininic acid was, as stated, *d*-arginine. In accordance with the more usual practice, the designation *d*-arginine has been used in our papers to refer to the "natural" enantiomorph.)

Results of Hydrolysis Experiments—The details are recorded in Tables I to IV.

TABLE II

Liver Arginase and Cobaltous Chloride with δ-Guanidinovaleric Acid and with Argininic Acid

Substrates, those of Table I and *d*-arginine nitrite; liver arginase solution, 2 ml.; phosphate buffer (1 M, pH 7.45), 5 ml.; cobaltous chloride added where indicated; water to 20 ml.; caprylic alcohol, 2 drops; digestion, 3 hours at 37°; urea estimated and results expressed as in Table I.

Experiment No.	$CoCl_2 \cdot 6H_2O$	Substrate*		0.02 N HCl neutralized	Substrate hydrolyzed
	mg.		*mg.*	*ml.*	*per cent*
1		*d*-Arginine hydrochloride	42	3.09	39
2	2	" "	42	6.41	80
3		" nitrite	44	3.10	39
4	2	" "	44	6.50	82
5		δ-Guanidinovaleric acid	32	0.00	0
6	2	" "	32	0.00	0
7		Argininic acid*	35	0.30	0
8	2	" "	35	0.33	0

* See foot-notes of Table I.

TABLE III

Jack Bean Arginase and Cobaltous Chloride with δ-Guanidinovaleric Acid and with Argininic Acid

Substrates, as indicated (see Table I); enzyme, crude jack bean urease, 0.1 gm. (or *crude* crystalline urease of comparable argininolytic activity); buffer, in Experiments 1 to 4, 5 ml. of phosphate (1 M, pH 7.45) and in Experiments 5 to 8, glycine (1 M, pH 9.50); cobaltous chloride added where indicated; water to 20 ml.; caprylic alcohol, 2 drops; digestion, 20 hours at 30°. After digestion, the mixture was diluted to 25 ml. and ammonia estimated in a 10 ml. portion as usual (see text). The results are expressed as in Table I.

Experiment No.	pH	$CoCl_2 \cdot 6H_2O$	Substrate*		0.02 N HCl neutralized	Substrate hydrolyzed
		mg.		*mg.*	*ml.*	*per cent*
1	7.45		*d*-Arginine carbonate	45	0.45	5
2	7.45	2	" "	45	7.76	89
3	7.45	2	δ-Guanidinovaleric acid	32	0.00	0
4	7.45	2	Argininic acid*	35	0.06	0
5	9.5		*d*-Arginine carbonate	45	2.16	25
6	9.5	2	" "	45	4.03	46
7	9.5	2	δ-Guanidinovaleric acid	32	0.07	0
8	9.5	2	Argininic acid*	35	0.44	0

* See foot-notes of Table I.

From the results given in Table I, it is seen that under conditions similar to those more usually employed in this series of investigations, *d*-arginine in the presence of liver arginase was extensively hydrolyzed in 20 hours in reaction mixtures of pH 7.45 or 9.5, while the hydrolysis of the closely related compounds, δ-guanidinovaleric and argininic acids was quite negligible. Similarly, the data of Table II emphasize that under conditions in which the arginase preparation used was normally activated in the presence of cobaltous ion so that *d*-arginine, introduced as hydrochloride or nitrite, was 80 per cent hydrolyzed in 3 hours at 37°, the two related guanidino compounds, in contrast, were not observedly changed. With jack bean argininolytic enzyme and cobalt chloride under conditions suitable for the extensive hydrolysis of *d*-arginine during 20 hours, there was observed no hydrolysis of the other two substrates (Table III).

Since, however, from a chemical standpoint enzyme specificity probably is not an absolute characteristic but rather one of degree, it was considered desirable to test the specificity of liver arginase with these closely related analogues of arginine under extreme conditions; *i.e.*, with 100 times the amount of enzyme usually employed together with cobaltous and manganous salts, in favorable pH regions, and during a long digestion period. These procedures appeared, in a measure to "force" the enzymatic hydrolysis of δ-guanidinovaleric and argininic acids. Table IV brings out that in 65 hours the former compound was hydrolyzed to the extent of 8 to 14 per cent and the latter, 25 to 38 per cent. Contrarily, there has been effected, in less than one-twentieth of this time, with a greatly diminished amount of enzyme, the hydrolysis of *d*-arginine to the extent of 80 per cent of its initial concentration. The significance of these results will be discussed later.

Hydrolysis of Guanidino Compounds in Alkaline Solution

Non-Catalysis by Certain Heavy Metal Salts and Hydroxides—The ions, Fe^{++}, Co^{++}, Ni^{++}, and Mn^{++}, which have been considered to participate through coordination in the enzymatic hydrolysis of *d*-arginine do not *of themselves* function as catalysts in this process. These ions alone appear to be entirely devoid of catalytic activity under the conditions of this series of investigations upon arginase. This is based upon the results of numerous

control experiments in which the enzyme was omitted in arginase-heavy metal salt studies. Equally without any striking effect are these ions or the hydroxides derived from them in the alkali-catalyzed hydrolysis of *d*-arginine. When, for example, there

TABLE IV

Specificity of Calf Liver Arginase: More Extreme Test with δ-Guanidinovaleric and Argininic Acids

Enzyme, liver arginase solution, 2 ml. (extract of 100-fold the dried arginase preparation usually used; *i.e.*, 11 gm. of powder extracted with 50 ml. of water); buffer, 5 ml., of such strength that in the final reaction mixture (10 ml.), ionic strength, μ, equaled unity: in Experiments 1 and 3, phosphate of pH 7.4 and in Experiments 2 and 4 glycine of pH 9.2; activating metal salt, $CoCl_2 \cdot 6H_2O$ (Co) or $MnCl_2 \cdot 4H_2O$ (Mn), 2 mg., as indicated; water to 10 ml.; caprylic alcohol, 0.1 ml.; toluene, 0.2 ml.; temperature 37°; time 65 hours. Digestion was carried out in aeration tubes and the estimation with urease was made for the urea in the entire mixture; the results are expressed accordingly. Substrates are as indicated (*cf.* Table I). In parallel experiments under similar conditions there was hydrolyzed 93 per cent of an amount of *d*-arginine equivalent to the argininic acid or δ-guanidinovaleric acid used in the tabulated determinations.

Experiment No.	Metal salt	Substrate*		0.02 N HCl neutralized†	Substrate hydrolyzed
			mg.	*ml.*	*per cent*
1	Co	δ-Guanidinovaleric acid	11.56	0.58	8
2	Mn	" "	11.24	0.99	14
3	Co	Argininic acid‡	13.1	1.90	25
4	Mn	" "	13.1	2.89	38

* Complete hydrolysis of the argininic acid would have produced urea ammonia equivalent to 7.60 ml. of 0.02 N HCl; for the δ-guanidinovaleric acid, 7.28 and 7.08 ml. of 0.02 N HCl for Experiments 1 and 2 respectively.

† The results have been corrected on the basis of appropriate controls. Under the conditions of these experiments the controls are larger than usual. The magnitude of the blanks is lowered when the procedure suggested in "Note on estimation of urea" is followed.

‡ See "Preparation of argininic acid" and Table I.

was added to a 0.005 M solution of *d*-arginine hydrochloride a nickelous or cobaltous salt followed by adjustment with sodium hydroxide to pH 8.5 or 9, after which the solution was permitted to stand at 37° for 3 weeks, a suitable test disclosed no hydrolysis of the guanidino acid. Moreover, manganous salts failed to effect

any hydrolysis of 0.008 M *d*-arginine dissolved in a 0.1 M glycine buffer of pH 8.5 when the mixture was held at 37° for 4 weeks.

The rate of hydrolysis of *d*-arginine or guanidine in an alkaline solution becomes appreciable above pH 10.5 even when the reaction is observed at 35-40°. Under such conditions there is no observable change in rate in the presence of nickelous hydroxide.

Hydroxyl Ion-Catalyzed Hydrolysis of d-Arginine and Other Guanidino Compounds—Observations were made with *d*-arginine hydrochloride, δ-guanidinovaleric acid, argininic acid, glycocyamine (guanidinoacetic acid), and guanidine. With each substance was prepared a solution 0.008 M with respect to the guanidino compound and 0.1 M with respect to potassium hydroxide. The solutions were kept in tightly stoppered, 1 liter Erlenmeyer flasks at 37.8° ± 0.2°. Because of the low rate of the hydrolyses, these conditions were considered adequate for comparative purposes. As controls were used a solution of KOH (0.1 M) and solutions of the following, each of which was *also* 0.1 M with respect to KOH: 0.008 M urea, 0.004 M ammonium sulfate (*i.e.*, the salt was added to the solution of KOH), 0.008 M *d*-ornithine, 0.008 M glycine. To each of the solutions were added caprylic alcohol, 0.3 ml., and toluene, 0.3 ml. Hydrogen electrode determinations of the pH initially and after the final hydrolysis measurements showed all the values to be pH 12.58 ± 0.02.

At selected intervals two 10 ml. samples were taken from each flask. One sample was used at once for the determination of ammonia by aeration. The second sample was subjected to treatment with urease before aeration, the result giving total ammonia (*i.e.*, free NH_3 + urea NH_3). For this purpose, the sample was brought to pH 7.5 with HCl; 6 ml. of phosphate buffer (1 M, pH 7.49) and 0.05 to 0.10 gm. of crude urease were added, after which the mixture was held 2 hours at 30°. This was followed by the usual aeration procedure. The presence of urea in the hydrolysis mixtures was shown also by tests with xanthydrol (crystallization of dixanthydryl urea).

Results—The analytical data are summarized in Table V. It is seen that the determinations of urea were sufficiently accurate and that there occurred little or no loss of ammonia from the reaction flasks. There was formed no ammonia in the urea, glycine, or *d*-ornithine controls. *Ammonia did appear in the reaction mix-*

tures. Its absence in the urea, glycine, and *d*-ornithine controls indicated that the ammonia formed during the hydrolysis of the guanidino compounds was derived from some source other than a secondary decomposition of urea or a residual amine. Accordingly, it appeared likely that in addition to the main reaction leading to urea and an amine, there was occurring also a simultaneous hydrolysis of the substituted guanidino compounds to yield ammonia and substituted ureas (*e.g.*, citrulline from *d*-arginine). A similar hydrolysis of a substituted guanidine by two paths was studied by Neubauer (15) who found (*cf.* (16)) that the decomposition of creatine by hot barium hydroxide gave sarcosine and urea together with methyl hydantoin and ammonia. Likewise, Schotté (17) found that creatinol, $H_2N—C(=NH)—N(CH_3)—(CH_2)_2OH$, with N alkali at room temperature yielded ammonia, urea, N-methyl-N,β-hydroxyethylurea, and N-methylaminoethanol. The data obtained in the present study have been analyzed on the basis of two concurrent reactions in each hydrolysis experiment. These statements are applicable to the substituted guanidino compounds and not to the case of guanidine, itself, of which the products of controlled hydrolysis are solely ammonia and urea.

Some data on the alkaline hydrolysis of guanidine were presented by Bell (18); calculations based on these data indicate that the process studied by him was essentially pseudomonomolecular. The enzymatic hydrolysis of *d*-arginine under certain conditions has also been shown (2, 3) to proceed pseudomonomolecularly. The data of the alkaline hydrolyses of this investigation (treated as irreversible reactions) were tested by substitution in a first order equation. The rate of conversion of a substrate compound is formulated as $-ds/dt = ks$, whence $k = (2.303/t) \times (\log a - \log s)$, where k = reaction constant, a = initial substrate concentration, and s = substrate concentration at time, t.

The reaction constant k would equal the sum of the constants for the two simultaneous hydrolyses, the rate of each of which is dependent upon the substrate concentration. Thus, $k = k_1 + k_2$, where k_1 and k_2 are the reaction constants for the processes that lead to ammonia and to urea, respectively. The *ratio* of these reaction constants should be directly proportional to the ratio of the molar equivalents of the respective products of hydrolysis formed in the two processes at any time, t.

TABLE V

Hydroxyl Ion-Catalyzed Hydrolyses: Determinations of Ammonia and "Total Ammonia"

Temperature, 37.8°; pH 12.58; all values for k are in inverse hours; k_1/k_2 shows average values of ratios of the relative rates for the two paths of hydrolysis; the last column, values of k from the slope of the curve in the graph of log s with respect to time.

The data for A. and T.A. are expressed as ml. of 0.02 N HCl neutralized by the NH_3 formed. A. is equivalent to ammonia directly formed; T.A. to total ammonia (free NH_3 + urea NH_3) (see text).

Substrate*	Initial amount in 500 ml.		Time, hrs. 0	28	48.5	91.75	141	215	354.5	522.25	691	$\frac{k_1}{k_2}$	k
	gm.												
d-Arginine hydrochloride	0.8432	A.	0.03	0.07	0.07	0.15	0.25	0.28	0.37	0.51	0.50		
		T.A.	0.05	0.48	0.86	1.58	2.22	3.21		5.36	6.05	0.21	0.00259
		$k \times 10^5$		250	258	268	263	268		254	248		
δ-Guanidinovaleric acid	0.6365	A.	0.06	0.04	0.04	0.15	0.19	0.24	0.32	0.40	0.38		
		T.A.	0.01	0.53	0.88	1.70	2.39	3.41	4.68	5.65	6.58	0.17	0.00286
		$k \times 10^5$		268	258	296	284	291	288	286	320		
Glycocyamine	0.4683	A.	0.05	0.10	0.08	0.22	0.44	0.50	0.67	0.92	0.97		
		T.A.	0.03	0.50	0.86	1.58	2.28	3.04	4.31	5.19		0.40	0.00275
		$k \times 10^5$		279	264	278	295	272	275	277			
Guanidine carbonate	0.3642	A.	0.05	0.53	0.88	1.01	2.07	2.67	3.34	3.49	3.86		
		$k \times 10^5$		512	507	485	511	509	499	382	459		0.00510
		T.A.	0.04	1.45	2.50	4.44	6.01	8.04	10.00	10.92			
		$k \times 10^5$		455	477	499	487	508	493	446			0.00499
Argininic acid	0.7032	A.	0.04	0.07	0.04	0.08	0.20		0.32		0.45		
		T.A.	0.04	0.59	0.92	1.76	2.42		4.45		6.32	0.16	0.00279
		$k \times 10^5$		311	278	288	285		261		281		

Urea, control	0.2395	A.	0.06	0.04	0.03	0.05	0.05	0.09	0.07	0.17	0.16		
		T.A.	7.75	7.74	7.85	7.96	7.78	7.93	7.86	7.78	7.92		
Ammonium sulfate, control	0.2650	A.	3.97	3.98	3.99	3.92	3.99		3.88	3.92	3.87		
		T.A.	3.81	3.93	3.88	3.92	3.82	3.96	3.90	3.88	3.89		
KOH, 0.1 N, control		A.	0.03	0.00	0.00	−0.01	−0.02	−0.02	−0.04	0.05	0.03		
		T.A.	−0.02	0.00	−0.01	0.01	0.02	0.02	−0.07	0.01	0.01		
Per cent†					98.7	99.7	96.9	99.9	100.1	99.0	99.9		

* The purity of these materials was determined by analysis.

† Per cent of urea estimated in parallel controls (4.5 mg. of urea) which were carried out with each series of determinations.

$$\frac{k_1}{k_2} = \frac{\text{moles } NH_3 \text{ formed}}{\text{moles urea formed}}$$

The analytical results (Table V) are given in terms of ml. of 0.0200 N HCl neutralized by ammonia. The values of a may be formulated conveniently in terms of ml. of 0.0200 N HCl equivalent to the amount of guanidino compound initially present in 10 ml. of a reaction mixture. For an 0.008 M solution, this would be 0.00008 mole = 4.00 ml. of HCl. Of a substrate, the moles

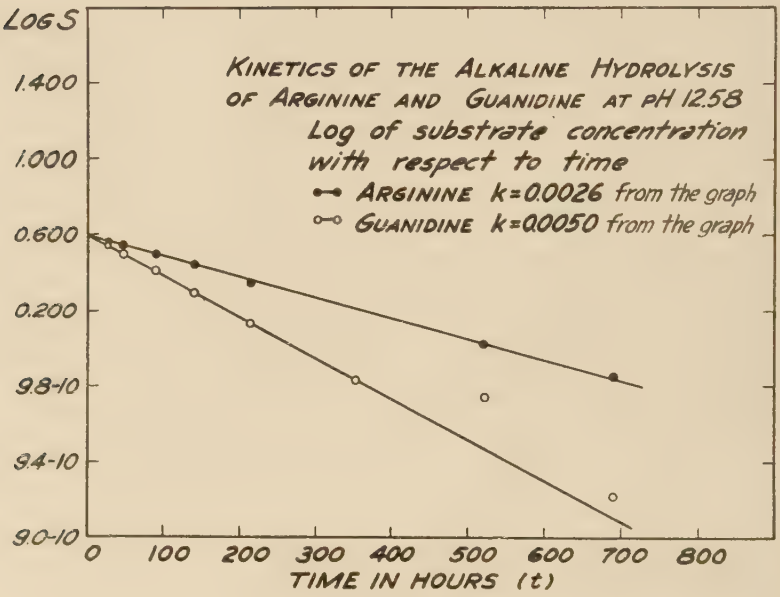


FIG. 2

hydrolyzed at time t are equivalent to the sum of moles of NH_3 and moles of urea formed; this may be given in terms of the *sum* of A. (Table V), the ml. of 0.02 N HCl neutralized by NH_3, and U., which is one-half the ml. of HCl neutralized by the urea NH_3 (obtained by urease hydrolysis). Thus, U. = $\frac{1}{2}$ (T.A.$-$A.) and $s=a$ $-$ (A. + U.). T. A. = "total ammonia" in 10 ml.

However, in the case of guanidine carbonate, a is taken alternatively as 4.04 ml. or as 12.11 ml., depending upon the choice of A. or T.A., respectively, as equivalent to moles of guanidine hy-

drolyzed at time t. This is permissible, since the hydrolysis of guanidine here may be considered to involve only the reaction

$$HN{=}C(NH_2)_2 + H_2O \rightarrow NH_3 + O{=}C(NH_2)_2$$

The data depicted in Table V and Fig. 2 disclose (1) pseudo-monomolecularity in the hydrolysis of each of the guanidino compounds, observed during a period of almost a month, (2) reaction constants (k) of the same order of magnitude in all cases (the magnitude of k for guanidine exceeding that for any of the substituted guanidines studied), (3) comparable ratios *for the δ-guanidino-substituted acids* of k_1/k_2, the reaction constants respectively for the ammonia- and urea-producing paths.

DISCUSSION

Certain aspects of enhancement of liver arginase activity in the presence of activating metal salts are emphasized in the several pH-activity curves (Fig. 1). In the presence of cobaltous or nickelous ion (but not of the potently activating manganous ion) there results a marked shift in the optimal pH zone for our preparations of liver arginase, connoting a rather wide extension of the range of effective action into the more acid region. We consider it not improbable that this phenomenon reflects, *in part*, the relative ability of the postulated enzyme-metallous ions concerned to form coordinate bonds, for example, with appropriate groups of the substrate molecules.[2] This may approximate the corresponding behavior of the effective heavy metal ions, themselves, which under certain conditions ((3) Fig. 3) have been found to follow, qualitatively, the order $Ni^{++} > Co^{++} > Mn^{++}$. Elsewhere, the matter has been elaborated in some detail (3). It is to be noted that relatively more emphasis is accorded the data summarized by the pH-activity curves *in the less alkaline region* than in the sections of higher pH value; this applies in

[2] The discussion neglects the question of the relative stability of postulated donor-enzyme-metal complexes in different pH regions; it neglects, further, the effect of their varying stability upon the activity-pH relations. It may be stated that unpublished dialysis experiments in this laboratory strongly indicate a significantly greater stability (at pH 7) for cobaltous- or nickelous-arginase than for manganous-arginase (Richards, unpublished experiments).

particular to the detail of the *manganous*-enzyme curve in the alkaline region exceeding pH 9, since the controls in this region gave rather large blanks.

The results of this study make apparent one factor underlying the differences in the pH-activity curves of arginase (and certain other (1) enzymes) as developed by different observers. Thus, the character and quantity of heavy metal activator ions present in the crude enzyme preparations employed must determine to some extent the characteristics of the activity curve. In consequence, it is not surprising that an activity curve (19, 20) evaluated for the arginase preparations used by Hunter and his collaborators resembles somewhat the arginase-manganous curve presented here (Fig. 1). Of pertinence to the discussion are the experiences of various workers. Hunter and Morrell first (21) reported an optimum at pH 7.2, but later (19) amended their conclusions and placed the optimum at about pH 9.8. Hino (22) found the optimum for his preparation at pH 7.3 to 7.5, while Edlbacher and Bonem (23) reported pH 9.5 to 9.8; Edlbacher and Simons later indicated an optimum near pH 9.0 (24).

The identity of the metallic component of, or coenzyme (1) that cooperates *in vivo* with, natural liver arginase is at present a matter of conjecture. There may be involved one or more of the heavy metal constituents of liver cells such as Fe^{++} or Mn^{++} or a metallo-organic entity.[3] Presumably arginase is a protein. Edlbacher and Pinösch (27) now stress the significance of manganous ion for its activation (*cf.* (5)).

Although intensive labor of fractionation will be required to gain further insight into the nature of arginase, the present position has permitted the construction of a useful working theory concerning a phase of its mode of action (1–3). Only certain aspects need be stressed here. The most effective *in vitro* activators

[3] The fact that *d*-ornithine in contradistinction to urea slows the rate of *d*-arginine hydrolysis in the presence of arginase is not surprising (*cf.* (25) p. 929). Practically, the hydrolysis is irreversible. The activity of the arginase *itself* doubtless is diminished by *d*-ornithine under certain conditions. A similar effect would not be anticipated for urea; suitable titrations demonstrate (26) that *d*-ornithine readily enters into metallo complex formation.

seem to be reduced ions of certain metals of the first transition series in the periodic table. The characteristics of inhibitions and reactivations of liver arginase suggest that catalysis by arginase involves metal-coordinated complexes. Furthermore, the observations concerned with titrations of *d*-arginine in the presence of certain heavy metal salts and the shifts in pH-activity curves together may be taken as an indication that the α-amino group of *d*-arginine may be concerned in the elaboration of dissociable catalyst-substrate complexes through metallo coordination. Elsewhere this has been depicted on a crude, tentative basis (1).

Our representation admittedly has been incomplete. For example, one requirement for effective arginase action appears to be a carboxyl anion in the substrate; esters of *d*-arginine are reported unattacked (7, 9, 23). It seems most probable that the carboxyl anion in addition to the α-amino group is involved in the orientation of substrate to metallo-enzyme, somewhat similarly to its involvement in simpler α-amino acid metallo complexes. In addition, any complete picture must account more adequately for the importance of geometric configuration.[4]

We have proposed (1, 2) that the modification of resonance ((25) p. 1872) in the guanidinium ion of *d*-arginine might account most fundamentally for the unique action of the enzyme arginase. Earlier we diagrammed this as resulting from the formation of a

[4] Compare the reported (28) non-hydrolysis of *dl*-ϵ-guanidino-α-aminocaproic acid (homoarginine). Arginase is not an effective catalyst in the hydrolysis of guanidine or certain simple derivatives (29), *including γ-guanidinobutyric acid* ((7, 23, 30), *cf.* (31) (25) p. 929)) regarding which conflicting statements have appeared. On the contrary, canavanine, $^{+}H_2N{=}C(NH_2){-}N(H){-}O{-}(CH_2)_2{-}C(NH_2)(H){-}COO^{-}$, which from a purely structural view-point is *d*-arginine in which a methylene grouping is replaced by hydroxylamino oxygen, is hydrolyzed (32) in the presence of a liver enzyme to urea and canaline. It is *conceivable* that the argininolytic enzymes are identical with the canavanases (*cf.* (3) foot-note 5). The properties of canavanases will be studied in this laboratory. Difficulties which have been experienced in the evaluation of results published by different authors in the field of enzyme specificity are often due to the omission of even a semiquantitative treatment of the relative efficiency of the enzyme with respect to the substrates considered.

coordinate link between metal ion and substituted guanidinium nitrogen. In the absence of supporting direct evidence we prefer now to submit only that the character of the resonance could be altered through some appropriate addition reaction involving the guanidino group and a grouping of the enzyme molecule. On this basis, the effect would involve a "weakening" of the bond shared by the ion's central carbon atom and the substituted nitrogen atom (*i.e.*, N attached to the side chain) and, possibly, a concomitant strengthening of the bonds with the other 2 nitrogen atoms, through intensified resonance involving them. An extended discussion of this and alternative possibilities regarding the mechanism of arginase action would be premature.

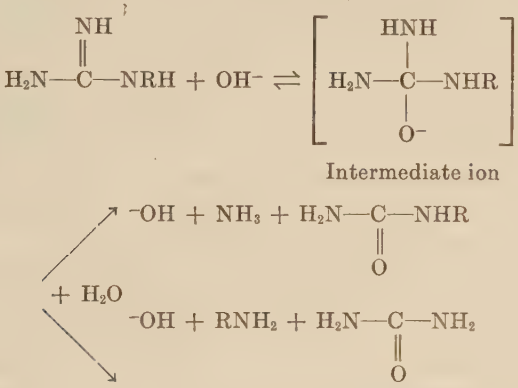
Assumptions concerning the rôle of the α-amino group of *d*-arginine in the *orientation of enzyme to substrate* are supported by tests of the specificity of arginase. The results recorded in the experimental part strengthen the opinion that the α-amino group is required for the *efficient* functioning of the enzyme. The catalytic efficiency of arginase with *d*-arginine, on the one hand, and δ-guanidinovaleric or argininic acid, on the other, is manifestly of a very different order. As between the latter two substrates the action of arginase with argininic acid seems slightly more noticeable, owing possibly to an enhanced associating effect attributable to the α-hydroxyl group.[5]

That arginase can be demonstrated, under extreme conditions, to display catalytic activity with these substances or with *l*-arginine (5) is not surprising in view of current concepts of enzymatic and general catalysis. For example, the postulated catalyst-substrate complex (*ES* in Equation 1) is considered to be the species chiefly undergoing hydrolytic change with regeneration of enzyme (*E*). If certain guanidino compounds structurally related to *d*-arginine may be assumed to enter into equilibria analogous to the process shown in Equation 1, the efficiency of the enzyme with respect to their hydrolysis may be related importantly to the magnitude of the association constants. Thus, with the use of a relatively large amount of enzyme and a sufficiently long

[5] It has been reported (7) that α,N-benzoylarginine is hydrolyzed under certain special conditions. Octopine is unhydrolyzed (33) in the presence of arginase or is noticeably attacked (34) after long reaction periods. The α-uramido derivative of *d*-arginine is attacked (20), but much less efficiently than the parent amino acid.

reaction time there might result measurable hydrolysis. Then in a special sense "specificity" of the enzyme becomes a matter of degree. The advantage of a dissociable intermediate complex in which metallo-enzyme has been bound through coordination to the substrate's α-amino group and carboxyl anion would reside not merely in the provision for favorable association of enzyme and substrate, but, further, in an accompanying strategic orientation of the enzyme's "working group" with respect to the guanidinium ion.

The comparison of an enzymatic hydrolysis with a somewhat comparable non-enzymatic process has proved instructive. The salts of guanidines are ordinarily highly resistant to hydrolysis, even at elevated temperatures. Contrarily, the parent bases are more or less readily hydrolyzed with hydroxyl ion as catalyst.



In this study, there was observed the controlled non-enzymatic hydrolysis of *d*-arginine together with the like hydrolysis of a number of other guanidines, none of which is readily hydrolyzed with arginase as catalyst. The results are given in the experimental section. In contrast to the striking specificity of the argininolytic enzymes, there has been revealed the similarity in the kinetics of alkaline hydrolysis of the entire series, including *d*-arginine. In contrast, also, to the experiences of a number of workers with the enzymatic process, which seemingly produces from *d*-arginine only urea and *d*-ornithine, there had to be considered two paths for the alkaline hydrolysis of substituted guanidines. This has been discussed earlier; it is illustrated by the accompanying dia-

gram which includes a schematic representation of the mechanism of the alkaline hydrolysis.

This raises a question whether, in addition to its other unique attributes, most conspicuously, the efficient catalysis of *d*-arginine hydrolysis in the regions of hydrogen ion activity where the substrate is otherwise notably "stable,"[6] arginase possesses the property of directing the course of the hydrolysis practically exclusively in one path. It may be stated that with specificity of this character our suggestions regarding a possible correlation of the enzyme activity with alteration of resonance in the guanidinium ion of *d*-arginine seem entirely consistent.

SUMMARY

1. Exploratory activity-pH curves for certain preparations of liver arginase have revealed in greater detail some of the aspects of the enzyme action in the presence of heavy metal activator ions. The results are considered to indicate an important source of divergence in pH-activity curves of certain enzymes as developed by different workers. It is proposed that the data *taken with other observations* suggest the participation of the α-amino group of *d*-arginine in the orientation of enzyme to substrate through metallo complex formation.

2. There has been investigated the specificity of arginase, particularly in relation to the rôle of the α-amino group of *d*-arginine. In this connection are described modifications in the preparation of δ-guanidinovaleric and argininic acids. The question has been raised to what extent enzyme specificity may be a matter of degree rather than an absolute property.

3. A comparison has been made of the characteristics of the enzymatic hydrolysis of *d*-arginine and of the controlled alkaline (non-enzymatic) hydrolysis of *d*-arginine, δ-guanidinovaleric acid, argininic acid, glycocyamine, and guanidine. The results have disclosed important distinctions in the differently catalyzed processes. The unique qualities in the action of arginase are discussed in relation to an idea which visualizes a correlation of the

[6] The stability to hydrolysis of the salts of guanidine and certain of its derivatives, in contrast to the parent guanidino bases, has been attributed to a high degree of resonance in the symmetrical guanidinium ions.

enzyme action with the alteration of resonance in the guanidinium ion of *d*-arginine.

BIBLIOGRAPHY

1. Hellerman, L., *Physiol. Rev.*, **17**, 454 (1937).
2. Hellerman, L., and Perkins, M. E., *J. Biol. Chem.*, **112**, 175 (1935–36).
3. Stock, C. C., Perkins, M. E., and Hellerman, L., *J. Biol. Chem.*, **125**, 753 (1938).
4. Hunter, A., and Dauphinee, J. A., *J. Biol. Chem.*, **85**, 627 (1929–30); *Quart. J. Exp. Physiol.*, **23**, 119 (1933).
5. Edlbacher, S., and Zeller, A., *Z. physiol. Chem.*, **242**, 253 (1936); **245**, 65 (1936).
6. Edlbacher, S., and Burchard, H., *Z. physiol. Chem.*, **194**, 69 (1931).
7. Felix, K., Muller, H., and Dirr, K., *Z. physiol. Chem.*, **178**, 192 (1928).
8. Steib, H., *Z. physiol. Chem.*, **155**, 278 (1926).
9. Calvery, H. O., and Block, W. D., *J. Biol. Chem.*, **107**, 155 (1934).
10. Felix, K., and Muller, H., *Z. physiol. Chem.*, **240**, 4 (1936).
11. Ackermann, D., Engeland, R., and Kutscher, F., *Z. Biol.*, **57**, 180 (1911).
12. Sakaguchi, S., *J. Biochem.*, Japan, **5**, 12 (1925).
13. Felix, K., and Muller, H., *Z. physiol. Chem.*, **174**, 102 (1928).
14. Hellerman, L., Cohn, M. L., and Hoen, R. E., *J. Am. Chem. Soc.*, **50**, 1716 (1928).
15. Neubauer, C., *Ann. Chem.*, **137**, 288 (1866).
16. Gaebler, O. H., *J. Biol. Chem.*, **69**, 613 (1926).
17. Schotté, H., *Z. angew. Chem.*, **39**, 677 (1926).
18. Bell, J., *J. Chem. Soc.*, 1213 (1926).
19. Hunter, A., and Morrell, J. A., *J. Soc. Chem. Ind.*, **43**, 691 (1924); *Quart. J. Exp. Physiol.*, **23**, 89 (1933).
20. Hunter, A., *Biochem. J.*, **32**, 826 (1938).
21. Hunter, A., and Morrell, J. A., *Tr. Roy. Soc. Canada*, **16**, sect. 5, 75 (1922).
22. Hino, S., *J. Biochem.*, Japan, **6**, 335 (1926).
23. Edlbacher, S., and Bonem, P., *Z. physiol. Chem.*, **145**, 69 (1925).
24. Edlbacher, S., and Simons, E., *Z. physiol. Chem.*, **167**, 76 (1927).
25. Gilman, H., Organic chemistry, New York, **2**, 929, 1872 (1938).
26. Stock, C. C., Dissertation, The Johns Hopkins University (1937).
27. Edlbacher, S., and Pinösch, H., *Z. physiol. Chem.*, **250**, 241 (1937).
28. Greenstein, J., *J. Organ. Chem.*, **2**, 480 (1937).
29. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London and New York (1930).
30. Thomas, K., Kapfhammer, J., and Flaschentrager, B., *Z. physiol. Chem.*, **124**, 101 (1923).
31. Thomas, K., *Z. physiol. Chem.*, **88**, 465 (1913).
32. Kitagawa, M., *J. Biochem.*, Japan, **11**, 265 (1929); **16**, 339 (1932).
33. Moore, E., and Wilson, D. W., *J. Biol. Chem.*, **119**, 576 (1937).
34. Akasi, S., *J. Biochem.*, Japan, **26**, 129 (1937).

VITA

The author was born May 19, 1910, at Terre Haute, Indiana. All his formal education prior to graduate study was obtained in Terre Haute, Indiana. He graduated from Garfield High School in 1928 and received in 1932 the degree, Bachelor of Science in Chemical Engineering, from the Rose Polytechnic Institute. During the years 1932–36 graduate work was taken at the Johns Hopkins University in the Faculty of Philosophy for the degree, Doctor of Philosophy; the first year being in the Department of Chemistry and the last three in the Department of Physiological Chemistry.

For the year 1936–37, while on leave of absence from the Faculty of Philosophy, the author was the John Archbold Fellow in Medicine at the Johns Hopkins Hospital.